

SHORT COMMUNICATION

Bringing spiders to the multilocus era: novel anonymous nuclear markers for *Harpactocrates* ground-dwelling spiders (Araneae: Dysderidae) with application to related genera

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Abstract. Multilocus approaches are essential for accurately recovering the evolutionary processes underlying species and population history. However, historical inferences in non-model organisms are still almost exclusively based on mitochondrial DNA due to the difficulty of obtaining multiple informative loci. Here, we use a genomic library based approach, to generate 15 novel anonymous nuclear markers (ANMs) from the ground spider *Harpactocrates globifer* Ferrández 1986. The ANMs cross-amplify and sequence well in the target species and its two closest relatives, and some of them also in the more distantly related *H. ravastellus* Simon 1914. Levels of nucleotide diversity of the ANMs within *H. globifer* ranged from 0.05% to 1.4% and average sequence divergence between close congeneric relatives from 0.02% to 13.9%, supporting the utility of these loci in population and species-level analyses. Moreover, a cross-species amplification screened in other spider taxa showed that some of the loci could also potentially be useful in more distantly related genera.

Keywords: Genomic library, nuclear loci, multilocus approaches, non-model organisms

Multilocus approaches based on unlinked markers, including both mitochondrial (mtDNA) and nuclear markers, are essential for an accurate reconstruction of the evolutionary processes underlying species and population history. Inferences based exclusively on mtDNA markers are hampered by processes such as sex-biased behaviors (e.g., sex-biased dispersal), low dispersal ability and small population sizes, incomplete lineage sorting, introgression or selective sweeps (Avice 2000; Hare 2001; Irwin 2002; Ballard & Whitlock 2004; Wilkins 2004; Kuo & Avice 2005). In recent years the realization that gene trees and species trees can differ markedly in closely related species has sparked the development of a new generation of methods for species tree inference, which rely strongly on the information provided by multiple markers (Maddison 1997; Edwards 2009). Moreover, many studies have shown that the statistical power of parameters such as divergence times, population size and rates of gene flow, rely on the number of loci used (see Brumfield et al. 2003; Brito & Edwards 2009 and references therein). Recent advances, both technological (see reviews of Hudson 2007; Thomson et al. 2010; Ekblom & Galindo 2011) and methodological (e.g., Hey & Nielsen 2007; Heled & Drummond 2010; Hey 2010; Yang & Rannala 2010), have greatly facilitated the implementation of multilocus analyses. Unfortunately, the scarcity of available genomic information makes finding multiple informative loci in non-model organisms a daunting task (Thomson et al. 2010). This is especially true for spiders, in which just a handful of mitochondrial and nuclear markers that are variable at the population and species level can be reliably amplified and sequenced (see e.g., Hedin 1997 for nad1; Maddison & Hedin 2003 for nad1, 16S and EF-1 alpha; Ayoub et al. 2007 for EF-1 gamma; Bidegaray-Batista et al. 2007 for cox1 and 16S-nad1 fragment; Bond & Stockman 2008 for 12S-16S fragment and ITS-1 and ITS-2; Vink et al. 2008 for Actin 5C; Garb & Gillespie 2009 for cox1, 16S-nad1 fragment and EF-1 alpha; Muster et al. 2009 for ITS-1, 5.8S rDNA and ITS-2; De Busschere et al. 2010 for cox1 and 28S).

Here, we report on a method to circumvent this limitation by isolating novel anonymous nuclear markers (ANMs) from a spider genomic library. Our target organisms are species belonging to the ground-dwelling genus *Harpactocrates* Simon 1914 (family Dysderidae) which inhabit the mountain ranges of the Sistema Central in the

Iberian Peninsula. From west to east, the species *Harpactocrates gredensis* Ferrández 1986, *H. globifer* Ferrández 1986 and *H. gurdus* Simon 1914 are found along the range, showing narrow, almost non-overlapping distributions. As with other species (about 13) in the genus, they are generally found at high elevation (above 1000 m) in temperate and moist forests, suggesting a preference for cool and humid environments. Preliminary phylogenetic and phylogeographic studies have shown that Pleistocene glaciations had a major role in structuring populations in *Harpactocrates*. These observations have led us to hypothesize that *Harpactocrates* species in the Sistema Central underwent population range expansion toward lower elevations during cooler periods, facilitating gene flow, whereas at interglacial periods they retreated to high elevation refuges, which then led to population fragmentation (e.g., Masta 2000; Knowles 2001; DeChaine & Martin 2005; Carstens & Knowles 2007b; Mardulyn et al. 2009; Schmitt 2009). With the aim of testing this hypothesis using a multilocus species/population approach, we have developed 15 novel ANMs from a genomic library of *H. globifer*, which cross-amplify and sequence well in the closely related species *H. gurdus* and *H. gredensis* and some of them in a more distant species *H. ravastellus* Simon 1914. Additionally, we have screened cross-species amplification in other spider taxa in order to evaluate the range and potential application of the novel markers (see below).

We constructed a genomic library from the pooled DNA of two *H. globifer* specimens. Total genomic DNA was extracted using SpeedTools Tissue DNA extraction kit (Biotools) and concentrated via ethanol precipitations. Genomic DNA (~10 µg) was digested with EcoRI restriction enzyme (50 µL total volume). The enzyme was selected following the recommendations of Carstens & Knowles (2006). The goal was to ensure that the enzyme did not cut the mitochondrial DNA into fragments smaller than 1.6 kilobase pairs (kb), thereby reducing the likelihood of cloning mtDNA fragments. EcoRI was selected based on the information provided by a complete mitochondrial genome of a new species of *Parachetes* Alicata 1964 (pending formal description, Pons, Bidegaray-Batista & Arnedo unpublished data), which is the sister genus to *Harpactocrates*. Digested DNA was visualized on a 1% agarose gel stained with ethidium bromide, and fragments between 0.8 and 1.5 kb in length

Table 1.—Primer sequences for fifteen anonymous nuclear loci developed from a genomic library of *Harpactocrates globifer*. Names indicate the loci, forward and reverse primers, PCR annealing temperature T (°C), size of amplicon and GenBank Accession No. of the original cloned fragment. Performance codes indicate successful amplification in the following species: *H. globifer* (1), *H. gurdus* (2), *H. gredensis* (3) and *H. ravastellus* (4), *Parachites romandiolae* (a), *Dysdera erythrina* (b), *Harpactea corticalis* (c), *Loxosceles rufescens* (d), *Troglohyphantes lucifuga* and *T. pedemontanus* (e), *Nemesia randa* and *Iberesia brauni* (f). PCR products resulting in double bands (db) or multiple bands (mb) are indicated as superscripts.

Locus	Primer sequences (5'-3')	T (°C)	Amplicon size (bp)	Accession no.	Performance
qgL1	F: AGACAGCATTCAGAGTCCAAGCG R2: GCCGAAATAGTTTGAAGCTCGTTTGCG	56	416	JN654497	1,2,3,4, a ^{mb}
qgL5	F: TGCCACAGCCCCACTAAAATAGG R: GCCAGGTTGCCAGTTAAAATCACG	58	424	JN654498	1,2,3,4, a, b
qgL10	F: AGCGACACATCCTTACCTGCGT R: GCGCATCTGGAGAGCCTTTGA	58	621	JN654506	1,2,3,4, a ^{mb} , e
qgL12	F: TGGCACAGCAGTGGCCAGAA R: CATGTCAACCGAATAGAAATC	55	617	JN654509	1,2,3
qgL21	F: ACGCCCGAACCGACCTTTGC R: ACGAGGGAGGTGCTAGAAGCG	58	580	JN654499	1,2,3, a ^{db} , f ^{mb}
qgL22	F: ACGATGCACCTCGAAATGGTCTG R: TTGGCGCGGAACCTCTCAGC	58	508	JN654511	1,2,3,4, a ^{db}
qgL25	F: TGCCACAGCCACCCCTATCC R: AGGCCAACCGGAAAGTCAAGC	59	309	JN654510	1,2,3,4, a, b, d
qgL26	F: TCCCGGGTCACTGTTGGGAAG R: TCCCAACGTGAACGAACCGC	57	586	JN654500	1,2,3,4, a ^{mb}
qgL28	F: GTCCCGTCGTCCGGGGTTTG R: GCCACCATGCTTTTGTGTCTTC	59	401	JN654508	1,2,3,4, a
qgL29	F: TGGACTCCCGTTTCAAGAGCG R: CCACGCTATAATGGGCCCAAGC	53	459	JN654502	1,2,3, a ^{mb}
qgL32	F: AGCCCAACATCCGTTTGACC R: CGTCGGCAAAAGGGACCACCC	58	567	JN654503	1,2,3,4, a ^{mb}
qgL33	F: ACGTGCCACACTCTCTCTTTTCG R: TGCCTGCTCGAATCAAAACCCAGC	58	343	JN654504	1,2,3
qgL34	F: CGCGTCACCATAGGCTCTTCGC R: AGTTCCGGTGTGTGGCCGAGC	58	408	JN654507	1,2,3, a ^{mb} , b ^{db}
qgL36	F: AGCGTAACGTAGGGCGTGCAG R: CGGCCGCTTGGAAAGGTGGTC	58	627	JN654501	1,2,3,4, a ^{mb} , b ^{mb} , f ^{db}
qgL38	F: TCACAGGCATACGAAAGCGCC R: GCCGAGCGTAGGCCTAGCATAAC	58	454	JN654505	1,2,3,4, a

were excised and purified from the gel using the kit QIAquick Gel Extraction kit (QIAGEN). Excised fragments were concentrated via ethanol precipitations at 8 ng/μL and cloned using the CloneJET™ PCR Cloning kit (Fermentas) and One Shot TOP10 competent cells (Invitrogen). Transformed cells were plated on agar plates containing ampicillin. Five hundred colonies were individually picked with a pipette tip and added directly to a 25 μL PCR mix containing the primers included in the cloning kit. Amplified inserts were visualized on a 1% agarose gel stained with ethidium bromide. Of the 500 amplified inserts, 206 that ranged in size from 300 to 1200 bp were selected for sequencing. Forty-one sequences were discarded because they were either identical to other sequences or did not sequence well. The remaining 165 sequences were locally blasted against the *Parachites* sp. mitochondrial genome to ensure that there were no mtDNA sequences among the selected inserts, and in all cases they turned out to be part of the nuclear genome. Subsequently, we performed nucleotide BLAST searches against the GenBank database to characterize sequences. Most sequences (160 out of 165) reported non-significant BLAST hits. Thirty-eight of these sequences were subsequently selected for primer design based on features that ensured that they were non-coding regions, such as the presence of multiple stop codons in each of the six reading frames, and avoiding poly-A or -G runs, which are difficult to sequence. The primer pairs designed were initially screened in a set of individuals consisting of one from each of the three target species (*H. globifer*, *H. gurdus* and *H. gredensis*) and one belonging to a more distantly related species (*H.*

ravastellus from the Pyrenees). Each primer pair was tested in a standard 25 μL PCR mix with the following conditions: 3 min at 94°C followed by 30 cycles of denaturation at 94°C for 30 s, annealing at three different temperatures of 52°C, 55°C and 58°C for 35 s, and extension at 72°C for 1 min, with a final single extension step of 72°C for 10 min. Of the 38 previously selected loci, 15 produced a single band product for at least one of the annealing temperatures tested and sequenced well in the three target species (Table 1). The annealing temperature of the PCR reaction was subsequently optimized (see Table 1), and the extension time was adjusted according to the length of the fragment.

Eight to 10 individuals of *H. globifer* from different populations were sequenced for each locus (Table 2) to investigate how informative the markers might be for population and phylogeographic studies. In addition, diversity indices and population statistics of the anonymous markers were compared with those of known mitochondrial genes and nuclear introns. With this aim, a mitochondrial fragment (*16S-nad1*) spanning the 3' half of the 16S rRNA ribosomal subunit (*rml*), the complete tRNA leu (*rmlL*), the 5' half of the NADH dehydrogenase subunit I (*nad1*) and the nuclear intron of the signal recognition particle 54-kDa subunit (*srp54*) were amplified and sequenced for 10 individuals of *H. globifer* and a single individual of *H. gurdus*, *H. gredensis* and *H. ravastellus*, respectively. The *16S-nad1* fragment was amplified with primer pairs LR-N-13398 (Simon et al. 1994) and N1-J-12350 (5'-CCTARTTGRCTAR-ARTTRGCRSATCARCCAATTG-3') and the *srp54* with SRP54f1

Table 2.—Overall diversity measures and statistics estimated across 15 anonymous loci, *srp54* intron and *16S-nad1* mitochondrial fragment for *Harpactocrates globifer*. Sample size (SS) indicates the number of individuals sequenced for each locus, in brackets the number of individuals used in the estimations considering the 0.9 probability threshold in PHASE. The length (L) in bp for each locus after sequences end-trimming and excluding sites with gaps. The lengths of indels are indicated as they occurred in each locus (N/A: not applicable, if indels were not observed). The number of segregating sites (S) and haplotypes (H), nucleotide diversity (π), minimum number of recombination events (R_M) of Hudson (1985), linkage disequilibrium statistic (ZZ) of Rozas et al. (2001), Tajima's D test (D) of Tajima (1989), and the HKA test of Hudson (1987). Not significant (ns) and significant (*) values at $P < 0.05$ of statistics after coalescence simulations.

Locus	<i>H. globifer</i>										HKA Test (<i>H. globifer</i> / <i>H. gredensis</i>) <i>srp54</i> vs. other locus
	SS	L	Indel length	S	H	π	R _M	ZZ	D		
qgL1	10 (10)	313	N/A	8	3	0.0031	0	0.0000	-1.9072 *	<i>P</i> = 0.4764 ns	
qgL5	10 (10)	342	N/A	5	4	0.0039	2 ns	0.0477 ns	-0.1917 ns	<i>P</i> = 0.5292 ns	
qgL10	9 (6)	540	1, 8	12	7	0.0057	0	0.1707 ns	-0.9653 ns	<i>P</i> = 0.7549 ns	
qgL12	8 (8)	542	1, 2	16	7	0.0071	0	0.2002 ns	-0.8083 ns	<i>P</i> = 0.2500 ns	
qgL21	10 (9)	496	1, 2	15	10	0.0088	1 ns	0.1057 ns	-0.0070 ns	<i>P</i> = 0.7649 ns	
qgL22	10(10)	429	3, 1, 2	16	5	0.0140	0	0.0360 ns	1.2410 ns	<i>P</i> = 0.4838 ns	
qgL25	10 (9)	223	6, 1	1	2	0.0009	0	0.1170 ns	-0.5290 ns	<i>P</i> = 0.2839 ns	
qgL26	10 (8)	512	N/A	2	3	0.0005	0	0.0000	-1.4979 *	<i>P</i> = 0.3564 ns	
qgL28	10 (10)	317	N/A	8	6	0.0068	0	-0.0570 ns	-0.1529 ns	<i>P</i> = 0.4341 ns	
qgL29	10 (9)	373	N/A	12	6	0.0070	1 ns	0.1465 ns	-0.9463 ns	<i>P</i> = 0.9255 ns	
qgL32	10 (9)	490	5, 11, 3, 10	21	5	0.0119	0	0.0665 ns	-0.1766 ns	<i>P</i> = 0.5201 ns	
qgL33	10 (10)	266	N/A	12	7	0.0142	1 ns	-0.0074 ns	0.4320 ns	<i>P</i> = 0.5290 ns	
qgL34	10 (10)	319	1	13	8	0.0108	0	0.0678 ns	-0.4578 ns	<i>P</i> = 0.8684 ns	
qgL36	10 (8)	552	N/A	11	6	0.0057	0	-0.0444 ns	-0.1786 ns	<i>P</i> = 0.5850 ns	
qgL38	10 (9)	366	N/A	5	3	0.0067	0	0.2154 ns	2.1431 ns	<i>P</i> = 0.3583 ns	
srp54	10 (10)	119	N/A	7	8	0.0165	1 ns	-0.0154 ns	-0.0165 ns	-	
16S-nad1	10 (10)	851	1	76	9	0.0237	7 ns	-0.0103 ns	-1.5465 ns	<i>P</i> = 0.1021 ns	

and SRP52r1 (Jarman et al. 2002). PCR conditions were as follows: 94° C for 2 min; 35× (94° C for 35 s; 45° C for 45 s for *16S-nad1* and from 45° C to 50° C for 35 s for *srp54*; 72° C for 45 s for *16S-nad1* and 35 s for *srp54*; 72° C for 5 min).

The allelic phase of heterozygotic individuals was resolved using the algorithms provided by PHASE (Stephens et al. 2001; Stephens & Donnelly 2003) and implemented in DnaSP v5 (Librado & Rozas 2009). In the case where direct sequencing detected multiple copies of different lengths, either as a result of heterozygote individuals or paralogy, the PCR products were cloned and four to eight colonies sequenced. Cloning results were compatible with alleles of different length (i.e., high sequence similarity) rather than paralogous copies, assuming that polymorphisms due to singleton mutations among sequenced colonies represented errors of the *Taq* polymerase enzyme or cloning artifacts (see Villablanca et al. 1998; Calderón et al. 2009; Cummings et al. 2010; Dawson et al. 2010).

The taxonomic range of applicability of the novel markers was evaluated by screening a sample of specimens belonging to groups at different hierarchical levels: *Parachetes romandiolae* Caporaccio 1949 and *Dysdera erythrina* Walckenaer 1802 (Araneomorphae, Haplogynae, Dysderidae, Dysderinae), *Harpactea corticalis* Simon 1882 (Araneomorphae, Haplogynae, Dysderidae, Harpactinae), *Loxosceles rufescens* Dufour 1820 (Araneomorphae, Haplogynae, Sicariidae), *Troglyphophantes lucifuga* Simon 1884 and *T. pedemontanus* Gozo 1908 (Araneomorphae, Entelegynae, Linyphiidae), and *Nemesia randa* Decae 2005 and *Iberesia brami* L. Koch 1882 (Mygalomorphae, Nemesidae). Two independent PCR amplifications were performed for each locus using the following touchdown cycle: 94° C 2 min; 10× [94° C for 35 s, 63° C for 35 s (-0.5° C/cycle), 72° C for 1 min]; 10× [94° C for 35 s, 58° C for 35 s, 72° C for 1 min]; 15× [94° C for 35 s, 52° C for 35 s, 72° C for 1 min]; 72° C for 10 min. Results of the amplification success are summarized in Table 1.

The average interspecific sequence divergence (p distance) of the anonymous loci calculated with DnaSP v5 ranged from 0.02% (qgL26) to 8.1% (qgL28) between *H. globifer* and *H. gurdus*, 0.41% (qgL26) to 6.9% (qgL33) between *H. globifer* and *H. gredensis*, 1.8%

(qgL25) to 11.1% (qgL1) between *H. globifer* and *H. ravastellus*, 0.39% (qgL26) to 8.5% (qgL28) between *H. gurdus* and *H. gredensis*, 1.3% (qgL25) to 13.9% (qgL28) between *H. gurdus* and *H. ravastellus*, and 2.4% (qgL25) to 9.4% (qgL1) between *H. gredensis* and *H. ravastellus*. Percent nucleotide diversity within *H. globifer* ranged from 0.05% (qgL26) to 1.4% (qgL33). Genetic diversity indices, recombination and linkage disequilibrium measures and neutrality tests were calculated for each locus in *H. globifer* using DnaSP v5. Results are summarized in Table 2. We applied the HKA test of Hudson (1987) to assess whether levels of polymorphism and divergence were correlated, as predicted by neutral theory. The test was performed by comparing polymorphism in *H. globifer* and divergence between *H. globifer* and *H. gredensis* in the intron *srp54* versus each of the 15 anonymous loci and versus the mitochondrial fragment *16S-nad1*. Neither recombination nor linkage disequilibrium were found at any locus. None of the HKA tests showed deviation from neutral predictions, although Tajima's D test showed significant negative values for the loci qgL1 and qgL26. The mitochondrial fragment *16S-nad1* was 1.4 times more variable than the intron *srp54*, and from 1.7 to 47 times more variable than the anonymous loci qgL33 and qgL26, which are the most and least variable ANMs, respectively.

This study reports the first ANMs ever designed specifically for spiders. These kinds of markers, however, have been isolated and extensively applied in other organisms; e.g., grasshoppers (Carstens & Knowles 2006), halibuts and killifishes (De Bruyn et al. 2010a, b), oysters (Hare et al. 1996), birds (Lee & Edwards 2008) and lizards (Rosenblum et al. 2007; Noonan & Yoder 2009). ANMs are promising markers for multilocus approaches at the population and species level of closely related and recently diverged species, as has been shown in recent studies (Carstens & Knowles 2007a; Carstens & Richards 2007; Knowles et al. 2007; Lee & Edwards 2008). The novel ANMs designed here are potentially useful not only for the target species, but also for more distantly related species in the genus (e.g., *H. ravastellus*), as well as for species in related genera (e.g., *Parachetes*, or even subfamilies (e.g., *Dysdera*, see Table 1). However, the

applicability of these novel markers is compromised at higher taxonomic levels. A significant and negative relationship between performance and evolutionary distance from the target species is also observed among the popular microsatellite markers (Primmer et al. 1996). Although the strategy implemented in our study greatly facilitates and speeds up the isolation of variable markers for populations and species, the advent of the so-called next-generation sequencing (NGS) methods promise to revolutionize the discovery of novel markers in non-model organism even further. In fact, the protocols used here for ANM isolation can be easily coupled with NGS methods, simply by replacing the cloning step, to generate literally hundreds or thousands of novel markers for spiders. Moreover, NGS allows sequencing of lots of markers from multiple individuals and populations simultaneously. No doubt NGS methods will spawn a whole new era for phylogenetic, phylogeographic and population genetic studies in spiders and their allies.

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